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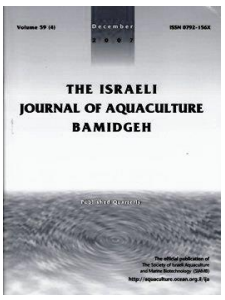

Digestive Enzymes of the Developing *Sepia pharaonis* Ehrenberg 1831 Paralarvae

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Key words: *Sepia pharaonis*, cuttlefish, cephalopod, digestive enzymes, ontogeny, paralarvae

Abstract

In order to understand the digestive physiology of *Sepia pharaonis* Ehrenberg 1831 (Sepiidae) paralarvae, and to formulate appropriate feeding strategies, the activity patterns of major digestive enzymes in relation to larval development were investigated. Trypsin, leucine aminopeptidase, alkaline phosphatase, alpha-amylase, and lipase were analyzed for 25 days from hatching. Results revealed that all digestive enzymes were present even prior to exogenous feeding, and each enzyme exhibited a distinct activity pattern. Leucine aminopeptidase, an enzyme involved in intracellular digestion, was found to be dominant and highly active at early developmental stages and declined with larval maturation. Lipase activity, involved in lipid metabolism, increased in the early stages of development but declined 10 days after hatching (DAH). Secreted enzymes (trypsin and amylase) and alkaline phosphatase (an indicator of larval gut maturity) exhibited low activity prior to 10 DAH and 13 DAH, respectively. However, these enzymes became highly active in the late developmental stages. These findings suggest the gradual gut maturation of *S. pharaonis* based on intracellular and secreted enzyme activities. The findings also indicate that paralarvae after hatching are well-equipped with digestive enzymes necessary to digest complex food items, and that the larval gut matures 13 to 20 DAH.

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Introduction

Cephalopod culture has been an increasing area of interest due to their rapid growth and high market value (Walsh et al. 2002; Garcia et al. 2004; Sykes et al. 2006). Their relatively short life span and high nutritional value increase the demand and make them a highly promising species for aquaculture as well as for biomedical fields (Boletzky and Hanlon 1983; Forsythe et al. 1991; Boal 1996). However, it has been reported that rearing at the early stages of development of some species such as *Sepia officinalis* is very delicate and problematic (Koueta et al. 2002; Domingues et al. 2003; Koueta and Boucaud-Camou 2003). This is also true for *Octopus vulgaris* (Villanueva 1995; Iglesias et al. 2004; Okumura et al. 2005). Development of hatchery techniques had been tried with limited success (Turk et al. 1986; Hanlon et al. 1987).

Common problems encountered in laboratory culture of this species are starvation at the time of yolk absorption and poor ingestion capacity (Vecchione 1987; Lee 1994). Knowledge of the digestive system ontogeny, especially food quality and acceptance, together with the physiological processes involved during food digestion and assimilation is vital for the development of effective feeding management and formulation of rearing protocols appropriate for the larvae (Chen et al. 2006).

There have been several studies on the functionality of the digestive tract through digestive enzyme assays in early cephalopod larval stages (Boucaud-Camou and Roper 1995; Boucaud-Camou and Roper 1998; Moltschaniwskyj and Johnston 2006; Pereda et al. 2009). However, these were conducted mostly in temperate climate cephalopod species. This study aims to evaluate the digestive ontogeny of a tropical cuttlefish, *S. pharaonis*, which is commonly found in the archipelagic waters of the Philippines. To the best of our knowledge, the present work on *S. pharaonis* paralarvae elucidates the changes in the digestive enzyme pattern for the first time.

Materials and Methods

Experimental animals. The experiment was conducted at the Multispecies Hatchery and Wet Laboratory of Zamboanga State College of Marine Sciences and Technology (ZSCMST), Fort Pilar, Zamboanga City, Philippines. Cuttlefish eggs were collected using squid pots on the east coast of Zamboanga City. Upon arrival at the hatchery, the eggs were incubated in a 40 L glass aquarium with water conditions suitable for cephalopod hatching, (35 ppt, 28°C). The incubated eggs were monitored daily until hatching. The species identity was confirmed as *S. pharaonis* by COI DNA sequence analysis.

Rearing conditions and collection of samples. Newly hatched paralarvae from the same batch of eggs were transferred into, and reared in, 50 L experimental tanks in a flow-through-system at a stocking density of 20 individuals/tank. The paralarvae were fed a mixed diet consisting of *Brachionus*, *Artemia* and mysids provided *ad libitum* from Days 0-5. Tilapia larvae were added as prey on day 6 onwards. The density of live prey was checked twice daily at 0800 and 1700 h.

Samples representing the developmental stage of *S. pharaonis* were obtained for digestive enzyme analyses. Twenty cuttlefish paralarvae at each particular larval stage were collected. Since cephalopods do not have clear and distinct larval stages, DAH was used as the index of their developmental stages. Larval collection was initiated upon hatching prior to the introduction of live prey (0 DAH) then continued progressively on 3, 5, 7, 9, 11, 13, 15, 20 and 25 DAH. Weight and mantle length of the samples were recorded (Table 1). The collected cuttlefish larvae were washed thoroughly with distilled water to remove excess salt and ink sacs with their contents. All samples were pooled and immediately stored at -20°C until analysis.

Table 1. Weight and mantle length of *Sepia pharaonis* hatchlings (mean ± SEM)

Stage (DAH)	*Weight (g)*	*Mantle Length (mm)*
0	0.115 ± 0.002	7.810 ± 0.109
3	0.116 ± 0.001	7.904 ± 0.098
5	0.143 ± 0.004	8.523 ± 0.112
7	0.161 ± 0.008	8.582 ± 0.090
9	0.197 ± 0.036	9.614 ± 0.194
11	0.284 ± 0.024	10.436 ± 0.124
13	0.359 ± 0.016	12.066 ± 0.170
15	0.365 ± 0.022	12.347 ± 0.274
20	0.388 ± 0.023	15.548 ± 0.133
25	0.536 ± 0.020	16.737 ± 0.356

Preparation of enzyme extracts. The frozen samples were thawed, weighed and homogenized in an ice-cold 50 mM citrate phosphate buffer (pH 7.0) at a ratio of 1:20 (wet tissue to volume) using Ultraturrax homogenizer. The homogenates were centrifuged at 4000 rpm at 4°C for 15 min. The supernatants were collected and used for enzyme assays within 4 h after homogenization. Trypsin, leucine aminopeptidase (LAP), alkaline phosphatase (AKP), alpha-amylase and lipase activities of *S. pharaonis* paralarvae were analyzed at 25°C and carried out in triplicate with corresponding blanks and control reactions.

Enzyme assays. Trypsin activity was measured according to the protocol of Kakade et al. (1969) using Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride as the substrate. The product formed was measured at an absorbance of 405 nm. L-leucine-p-nitroanilide was used as substrate on the analysis of LAP by reading its absorbance at 405 nm as described by Serrano and Traifalgar (2012). Alkaline phosphatase activity was assayed using the methods of Nigam (2007) with modifications utilizing p-nitrophenyl phosphate as substrate. The absorbance was measured at 420 nm. Further, alpha-amylase activity was determined following the method of Bernfield (1951) as modified by Areekijseree et al. (2004) using starch as substrate and read at 546 nm. Lipase activity was assayed using p-nitrophenyl laurate as substrate and reading the absorbance at 420 nm (Pinsirodom and Parkin 2001). Protein content of the extracts was determined following the methods of Bradford (1976) using bovine serum albumin as a standard at 595 nm. The concentrations of the assay products were determined based on the standard curve absorbance vs. product concentration. The enzyme activity was expressed as units/mg protein.

Results

All digestive enzymes measured were found to exhibit initial activity after hatching prior to the first feeding (Figs. 1-5). Proteolytic enzyme activities were active in the early developmental stages. As in other cephalopod larvae, tryptic activity was found to be minimal after hatching and the level of enzyme activity was consistently low until day 9. Progressive increase in tryptic activity is exhibited from 13 DAH until 25 DAH (Fig. 1). Similar to tryptic activity, LAP activity was found minimal after hatching but exhibited a notable pattern of increased activity starting at 3 DAH until 9 DAH. The LAP activity pattern remained stable exhibiting a steady state starting from 9 DAH until 20 DAH and a decrease in activity was noted at 25 DAH (Fig. 2). The activity pattern of AKP was similar to that of the other measured enzymes, exhibiting a low and constant pattern after hatching until 13 DAH. Thereafter, there was a marked increase in activity as larval development progressed until 25 DAH (Fig. 3).

The pattern of amylase activity exhibited a gradual but notable increase as the larva matured (Fig. 4). Lipase activity gradually increased after hatching reaching a peak at 9 DAH. The pattern of lipase activity remained stable from 9 DAH until 20 DAH and a sharp decline was noted at 25 DAH (Fig. 5).

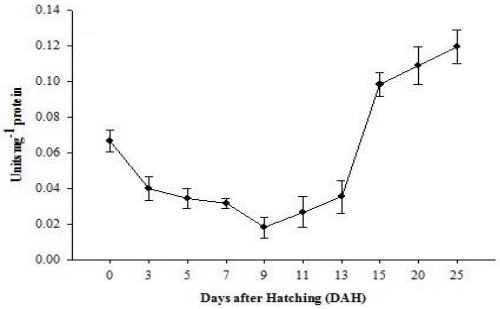

Fig. 1.Trypsin activity of *S. pharaonis* paralarvae (mean ± SEM).

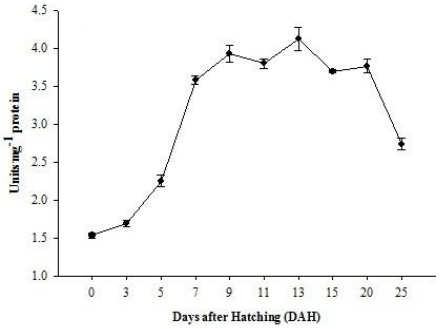

Fig. 2. Leucine aminopeptidase activity of *S.pharaonis* paralarvae (mean ± SEM).

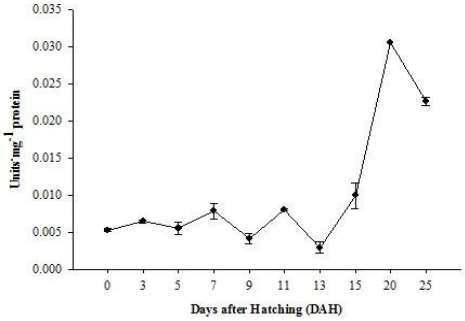

Fig 3.Alkaline phosphatase activity of *Sepia pharaonis* paralarvae (mean ± SEM).

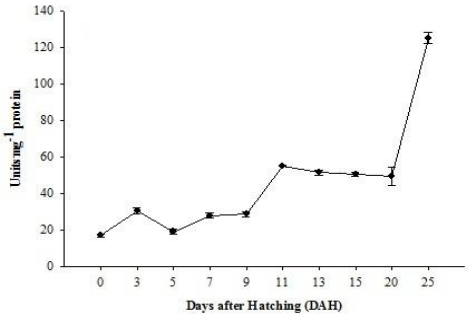

Fig 4. Amylase activity of *Sepia pharaonis* paralarvae (mean ± SEM).

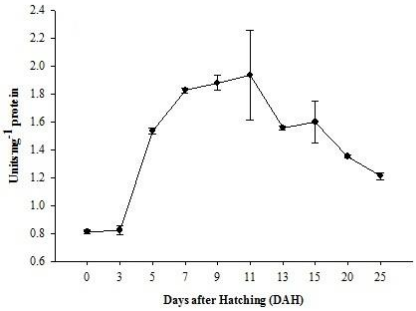

Fig. 5. Lipase activity of *Sepia pharaonis* paralarvae (mean ± SEM).

Discussion

It is important to understand the larval digestive physiology when developing feeding strategies appropriate for larval needs. Activity of major digestive enzymes in relation to larval development has been suggested as a key indicator of the rate of development and gut maturity (Zambonino Infante and Cahu 2001, Johnston et al. 2004). The present results suggest that *S. pharaonis* paralarvae are well-equipped with digestive enzymes necessary to utilize complex nutrients of prey organisms soon after hatching. Digestive trypsin activity upon hatching was at a minimal level until 13 DAH and a significant rise in activity was noted until 25 DAH. Similar to the present findings, low tryptic activity at early developmental stage and the notable rise of activity at late developmental stages have been also observed in several decapod larvae including *Macrobrachium rosenbergii* (Kamarudin et al. 1994), *Penaeus setiferus* postlarvae (Lovett and Felder 1990) and *Penaeus indicus* postlarvae (Ribeiro and Jones 2000). The sharp increase in tryptic activity in crustacean larvae has been attributed to the enlargement and maturation of the hepatopancreas as development progresses.

In *S. officinalis* paralarvae, tryptic activity was found to be highly active only 10 days post hatch. This conforms with the findings of the present study. Further, tryptic activity was not influenced by diet indicating that it is developmentally regulated (Koueta et al.

2000). Similar to the present findings, early stage *O. vulgaris* larvae exhibited low trypsin activity but this increased progressively reaching a peak at 7 DAH (Villanueva et al. 2002, Morote et al. 2005). In *S. officinalis*, early detection of digestive enzyme activity has been attributed to the presence of functional Boules cells, the secretory organ for digestion, upon hatching (Lacoue-Labarthe et al. 2010). Although, evidence suggests that the cephalopod larvae digestive gland is active at the onset of hatching, the secretion pattern and cellular development of this gland in relation to age is yet to be elucidated in *S. pharaonis*.

LAP is a membrane-bound enzyme involved in intracellular digestion and is highly active prior to the maturation of organs involved in extracellular enzymatic digestion (Zambonino Infante and Cahu 1994). In the present study, the increasing activity of LAP with progressing developmental stage while trypsin activity is at a minimum indicates that intracellular membrane-bound digestion is active at this earlier stage. Further, as LAP activity reached a plateau at 7 DAH this coincides with the initial rise of tryptic activity suggesting the onset of secretory and extracellular digestion. The present findings corroborate the documented ontogeny of the octopus digestive gland wherein after hatching (1 to 10 days), intracellular digestion of yolk reserves dominates for the generation of metabolic energy (Martinez et al. 2011). As the larvae develops, LAP activity decreases (>12 days post-hatch) as full functionality of the digestive gland manifests and complex prey food materials are utilized. Maturation of the digestive gland indicates full functionality of extracellular digestion and decline in LAP activity at 25 DAH in the present study, and may indicate digestive gland maturation in *S. pharaonis* larvae.

Alkaline phosphatase (AKP) is a membrane-bound enzyme at the brush border membrane of the intestinal villi. In most aquatic animal larvae studied, the rise in activity of AKP in the digestive tract indicates gut maturation since efficient brush border mode of nutrient absorption is a character of an adult organism with fully developed extracellular digestive capabilities (Henning et al. 1994). In the present study, AKP exhibited minimal activity at earlier stages and increased in activity at 15 DAH until 25 DAH. The pattern of AKP activity in the present study corroborates that observed in larval fish where a sharp rise in AKP activity indicated gut maturation at 3 weeks after hatching (Zambonino Infante and Cahu 1994; Ribeiro and Jones 2000). The pattern of increasing AKP activity at 13 DAH in the present study concurs with the pattern observed in larval fish however in a shorter period (Zambonino-Infante and Cahu 1994). This difference may be due to the long period of embryonic development of cephalopods prior to hatching where the digestive gland of cephalopods is well-developed and functional upon hatching. The rise in AKP activity from 13 DAH onward indicates gut maturation of *S. pharaonis* at this period.

Low alpha-amylase activity during early hatchling stages suggests that *S. pharaonis* paralarvae are carnivorous (Kamarudin 1994). In wild planktonic cephalopod paralarvae amylase activity in the digestive gland was absent implying that the larvae are highly carnivorous (Boucaud-Camou & Roper 1995). Similar to the present findings, amylase activity was low in *S. officinalis* larvae and this enzyme could only be detected on 10 DAH (Koueta et al. 2000). In the larvae of *Scylla serrata* (Serrano and Traifalgar 2012) and *Homarus americanus* (Biesiot and Capuzzo 1990) amylase activity was minimal at the early larval stages but as development progressed towards metamorphosis, amylase activity increased. Similar to other carnivore larvae, the significant increase in amylase activity starting at 9 DAH as observed in the present study may indicate a shift in feeding strategy from carnivory to omnivory as *S. pharaonis* larvae mature. This is probably a natural strategy towards energy efficiency and improvement of larval survival.

Sepia pharaonis larvae lipase activity increases as larval development progresses, exhibiting a peak at 7 DAH remaining at this level until 11 DAH then gradually declining until 25 DAH. The present result is comparable to the ontogenetic pattern of lipase activity observed in *M. rosenbergii* which exhibited higher levels in the early larval stages which declined as the larvae matured to the juvenile stage (Deru 1990; Kamarudin et al. 1994).The esterase peak activity, coincided with the increase in hepatopancreas size of this crustacean (Kamarudin et al. 1994). Similarly, in larval *Octopus bimaculoides* the

pattern of lipase activity increased at 5 DAH reaching a peak at 10 DAH and thereafter declining to the initial level of activity (Koueta et al. 2000). In the present study, the progressive increase in lipolytic activity during the early stages of this cephalopod paralarvae could be attributed to the increased utilization of yolk lipid reserves in the digestive gland (Boucaud-Camu and Roper 1995). The decrease in lipase activity at around 11 DAH could be due the digestive gland maturation (increase in size) and the depletion of oil reserves, similar to those observed in crustaceans and in octopus paralarvae (Martinez et al. 2011). The ultrastructure development and the biochemical composition of the digestive gland were not evaluated in the present study. This necessitates further investigation.

The present results suggest that *S. pharaonis* paralarvae are well equipped with digestive enzymes necessary for digestion and utilization of complex food nutrients. Each of the digestive enzyme measured indicate a distinct pattern that coincides with the larval development. Activity pattern of the intracellular enzymes, LAP and AKP, indicate that *S. pharaonis* paralarvae reach full gut maturity at around 13 DAH to 20 DAH. The present findings would be helpful in understanding the larval digestive physiology of this species and the development of feeding strategies to maximize larval production in aquaculture conditions by establishing a standard rearing protocol. Furthermore, the use of high protein diet that is costly can be minimized as it has been observed that this species becomes omnivorous as it matures. The present findings lead to the possibility of using formulated supplemental diets for cuttlefish paralarvae at day 13 since the larval gut is sufficiently mature and capable of hydrolyzing complex dietary ingredients.

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